CULTIVATION OF PASTEURELLA TULARENSIS ON A SIMPLE LIQUID MEDIUM

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CULTIVATION OF PASTEURELLA TULARENSIS ON A SIMPLE LIQUID MEDIUM

[Following is a translation of an article by Bohumir Lukas [see Note] of the Chair of Epidemiology of the Military Medical Research and Training Institute of J. Ev. Purkyne, Hradec Kralove, in Ceskoslovenska Epidemiologie, Mikrobiologie, Immunologie (Czechoslovak Epidemiology, Microbiology, Immunology), No 11, Prague, July 1962, pages 246-253.]

([Note]. With the technical collaboration of A. Chy'lova' and V. Simerkova').

The cultivation of Past. tularensis on liquid media remains to be a difficult and complex problem whose solution has been already attempted by many workers. In the year 1928 Suvorov et al, and Francis (6) described cultivation on beef broth with serum or ascites fluid. Later, however, Francis (5) had a more skeptical view of the possible growth of Past. tularensis on a liquid medium. In the following years Tamura and Gibby (19, 4), Miles et al, (12, 10) wrote about relatively complex synthetic or semisynthetic media (based on the hydrolysate of casein or gelatin, amino acids and growth extracts). Recently, Traub et al, (21) studying metabolism of Past. tularensis proposed a medium composed of 13-16 amino acids. A relatively simple liquid medium for the cultivation of tularemia was prepared by Steinhaus et al (17), Eigelsbach, et al (3) using GCBA media -- and Snyder et al (16) who achieved growth on a medium containing only peptone, glucose, NaCl and eventually thioglycoll acid.

The goal of our work was to utilize our experiences and findings obtained during the preparation of solid agar media (10) and to attempt the elaboration of a simple liquid cultivating medium.

Materials and Methods

Strains

The media tested were inocculated with strains 4 Ma, 098, 211, 130 (10). All of the strains used were virulent for white mouse (MLD-1 -- 100 cells) and guinea pig (MLD 10-1000 cells).

Chemicals

The media were prepared from the following chemicals: Bactopeptone Difco, Peptone Czechoslovak Organofarma, sodium thioglycollate, l cystein H Cl, nicotinic acid Difco, amide of nicotinic acid Difco, calcium pantothenate Difco, vitamin B_1, B_2, B_6 (supplied kindly by the Research Institute of Pharmacy and Biochemistry in Prague), glucose, NaCl.

Preparation and composition of media

A. Base medium
0.1 - 2.0% peptone Difco or Czechosl. Organofarma
0.5% glucose
0.5% NaCl
distilled water added. pH 7.5-4 before autoclaving or filtration.

The base medium was enriched by components presumed significant for the growth of Past. tularensis e.g.

B. (See Note**) 0.1 - 0.8% Sodium thioglycollate

C. (See note**) 2 - 4 mg% nicotinic acid
2 - 4 mg% amide of nicotinic acid
90 - 180 gamma% Ca pantothenate

D. (See note**) 2.5 gamma % vitamin B_1
15.0 gamma % vitamin B_2
7.5 gamma % vitamin B_6

Media containing base and components B, C, D were sterilised by passage through glass filter G 5.

(Note: The components were added to base A and eventually base A and B either singly or in various combinations.)

Medium TKGL (thioglycollate of sodium, blood, glucose, liquidum).

1.5% Peptone Czechosl. Organofarma
0.5% Glucose
0.5% NaCl
0.4% Na thioglycollate
distilled water added. pH 7.4 to 5 before autoclaving. Before inocculation 2 to 5% of defibrinated rabbit blood are added.

The liquid media were placed into test tubes (5 ml each), Ehrlermayer flasks or Patocka's hemocultivation bottles (with glass beads).

Medium TKGA (thioglycollate of Na, blood, glucose, agar).

1.5 - 2.0% agar strand
1.0% Peptone Czechosl. Organofarma
1.0% glucose, NaCl
5 - 10% defibrinated rabbit blood

Medium TKGAJ (Sodium thioglycollate, blood, glucose, agar, liver extract).

This medium has the same composition as TKGA and is merely supplemented by the liver extract. The preparation of solid media has been described in detail in another article (10).

Francis' medium and GCBA medium were prepared according to prescriptions listed in literature (7, 13).

Preparation of inoculum and method of inoculation

As inoculum we have utilised a 24 hour suspension washed off egg media. Density was measured nephelometrically (Pulfrich-blue filter, comparative glass No 1) and by the number of colonies that grew on TKGA medium from a 10^{-6} to 10^{-10} diluted inoculating suspension (the concentrated basic suspension, 10^{0} diluted, contained about 5 milliards of cells). The inoculum containing roughly 10^{8} - 10^{1} organisms was inoculated by a hypodermic in quantities of 0.1 ml to 3 to 5 test tubes filled with the medium to be tested. The media were incubated in a thermal chamber at 37°C for the period of 10 days.

Evaluation of growth

The growth of Past. tularensis on liquid media containing rabbit blood was evaluated according to the opaqueness produced, measured on the nephelometer (Pulfrich - green filter, comparative glass No 4) and the number of colonies determined by serial decimal dilution of a sample of the medium tested in a physiological solution (medium tested - dilution 10^{0}). Dilution of 10^{-4} to 10^{-6} was inoculated in the volume of 0.2 ml on 2 to 3 TKGA media. Growth on media not containing blood was determined by macroscopic evaluation of the opaqueness produced and [illegible] of the colonies on TKGA carried out in a way similar to that described above. The growth on the individual samples was evaluated each day and final results were read on the 9th or 10th day. As a control we used the opaqueness at 0 hours of incubation (immediately after inoculation) and [illegible] without inoculum.

Results

[illegible] our experiments we have verified some [illegible] media e.g. the effect of peptones and [illegible] on the growth of Past. tularensis.

Beside [illegible] we have studied first the growth of tularemia [illegible] concentration of Bacto peptone Difco and Organo-[illegible] (Table 1).

The table shows that Past. tularensis can be cultivated even in a very "poor medium." However, the success of the cultivation depends [illegible] the number of microbes inoculated, the quality and quantity of the peptones. Beginning with a certain concentration both of the peptones

used as preventive effect on growth; in Difco peptone from 2% concentration upwards and in Organofarma peptone from 1% concentration upwards.

Table I

Effect of Peptones on Growth of Past. tularensis

Inoculum [1]	Type of Peptone	Concentration of peptones in % 0.1	0.5	1.0	2.0
5×10^7	DIFCO	0	+ [2]	+	±
	ORGANO	0	+	0	0
5×10^6	DIFCO	0	+	+	±
	ORGANO	0	±	0	0
5×10^5	DIFCO	-	+	+	0
	ORGANO	-	0	0	-
5×10^4	DIFCO	-	0	0	0
	ORGANO	-	0	0	-

1) Strain 4 Ma
2) Evaluation of growth: ± very light opaqueness, + = weak opaqueness -10^8 -10^9 organisms /1 ml, ++ = strong opaqueness -10^9 -10^{10} organisms /1 ml, +++ = diffuse opaqueness -10^{10} and more organisms /1 ml, 0 = negative growth, - = experiment was not carried out.

On the peptone Difco tularemia multiplied from an inoculum linearly about 1000 times smaller than on the peptone Organofarma.

Table 2

Effect of Thioglycollate on Growth of Past. tularensis

Inoculum [1]	Thioglycollate Concentration in %	Peptone DIFCO [2] concentration in % 0.5	1.0
5×10^5	0	-[3]	+
	0.1	+	++
	0.2	++	++
	0.4	±	++
	0.8	0	++
5×10^4	0	0	0
	0.1	0	0
	0.2	++	-
	0.4	0	++
	0.8	-	+
5×10^3	0	0	0
	0.1	+	0
	0.2	++	-
	0.4	-	++
	0.8	0	+

1) Strain 4 Ma
2) The table does not include media with peptone Organofarma all of which had negative -O- growth.
3) Evaluation of growth: $\pm$ = very light opaqueness, $\pm$ = weak opaqueness (10^8 - 10^9 organisms /1 ml), + = strong opaqueness (10^9-10^{10} organisms /1 ml), +++ = diffuses opaqueness (10^{10} and more organisms /1 ml), O = negative growth, - = experiment was not carried out.

The base medium A containing peptone Difco and enriched with sodium thioglycollate had substantially better growth characteristics as can be seen from growth obtained even from reduced members of organisms inocculated. We were not able to record similar effect with media containing peptone Organofarma although we used large inoccula (Table 2).

Table 3

Effect of Defibrinated Rabbit Blood on Growth of Past. tularensis From Small Inoccula When Varied Concentrations of Peptones and Sodium Thioglycollate Were Used in the Medium

Inocculum [1]	Peptone Conc. in %	Thioglycollate Conc. in %	Growth on Media With Peptone [2] DIFCO	ORGANOFARMA
10^3	0.5	0.1	1×10^8	-
		0.2	3×10^8	7×10^7
		0.4	-	5×10^8
		0.6	-	3×10^7
	1.0	0.1	2×10^9	-
		0.2	1×10^{10}	8×10^8
		0.4	4×10^9	2×10^9
		0.8	1×10^9	1×10^8
10^2	0.5	0.1	1×10^9	-
		0.2	1.5×10^9	1×10^7
		0.4	-	7×10^7
		0.6	-	1×10^7
	1.0	0.2	4×10^9	-
		0.4	6×10^9	2×10^9
		0.8	3×10^8	7×10^8
10^1	0.5	0.1	1×10^8	
		0.2	1.5×10^8	0
		0.4	-	1×10^7
		0.6	-	0
	1.0	0.2	3×10^9	
		0.4	4×10^9	5×10^8
		0.8	1.2×10^9	7×10^7
10^3	2.0	0.1 cystein dzzo		0^3 1×10^9 [4]

1) Strain 4 Ma
2) Growth is expressed by number of cells per 1 ml.
3) Medium without rabbit blood (modified after Eigelsbach)
4) Medium with rabbit blood (modified after Eigelsbach)

In the table there is indicated also a certain relationship between the optimal amount of sodium thioglycollate and the concentration of peptone in the medium (See also Table 3).

In many subsequent experiments we have attempted to improve the culture medium by factors that -- according to the literature -- affect the growth of tularemia (12, 13, 14, 18, 19, 20). Because of this we added to the base A and B, either singly or in combination, nicotinic acid, amide of nicotinic acid, Ca pantothenate, vitamin B_1, B_2, B_6. Our efforts were practically in vain. Even with media prepared from yolk filtrates or liver extracts growth occurred only when mass inoculum was used. This growth was not standard and there were many cases of contamination on yolk media.

On the basis of our experiences with agar media we have therefore turned our attention to blood and its components. In many experiments we supplemented the base A and B (peptone, glucose, NaCl, sodium thioglycollate) with 2 to 5% of defibrinated rabbit blood. The results are shown on Table 3.

It is obvious that the addition of rabbit blood to media using both Difco and Organofarma peptones fascilitated primarily the reduction of inoculum to almost single organisms, and increased the intensity of multiplication especially in case of Difco peptone. Similar effect was observed with media modified after Eigelsbach, containing peptone Organofarma and cystein. Growth in liquid media appeared within 48 to 72 hours (diffuse opaqueness) [See Note7. Rabbit blood can be replaced with the same effect with human or guinea pig, but not sheep blood. Rabbit blood extract, filtrate or serum had lesser effect on the growth than whole blood.

([Note7: At first the added blood made the medium somewhat opaque, however after a few hours there developed a sediment above which there was a clear supernatant which became diffusely opaque due to growth of the organisms).

Table 4

Isolating Characteristics of TKGL in Comparison With Solid Agar Media

	TKGL	TKGA	TKGAJ	GCBA	Francis Difco	Francis Organo	McCoy Chapin
Liver	10/9*)	5/4	10/7	7/5	6/0	3/0	4/3
Spleen	9/8	4/3	9/8	6/4	5/2	2/0	3/2
Marrow	3/2	2/1	3/1	3/1	3/0	2/0	2/0
Blood	6/5	5/4	6/5	6/5	4/0	2/0	4/2

Strains: 098, 211, 130
*Numerator = number of cultivations, nominator = number of isolations of Past. tularensis.

On the basis of these results we have prepared a simple liquid TKOL medium (for preparation see Methods) which we tested in two series of experiments.

In the first series of experiments we have verified the isolating and culturing properties of TKOL on mice and guinea pigs infected with strains 098, 211, 130. From the organs (liver, spleen, marrow, blood) of the infected mice and guinea pigs we have prepared a 10% suspension which we inoculated into 5 ml of medium TKOA /sic/ and on solid media TKOA, TKOAJ, OCBA and Francis'. Within 72 or 48 and in some cases 24 hours we were able to observe growth on the liquid medium. This was verified by transfer inoculation onto solid medium TKOA (Table 4).

Table 5

Isolation of Past. tularensis From Blood Cultures (g.-pigs) Carried Out on Liquid and Solid Media TKOA

Strain	Dosage in MLD	Number of Positive Isolations in % Per Medium	
		Liquid TKOL	Solid TKOA
4 Ma	10	57	44
	1000	80	32
211	10	62	39
	1000	76	49

In the second part of the verifying experiment -- in direct blood cultures from guinea pigs -- we have utilized independent inoculum as supplementary blood on one side and as an advantage of the fact that larger volume of inoculum can be introduced into liquid media than on solid media, on the other.

The experiment was carried out on 80 g.-pigs divided into 4 groups of identical numbers. The test animals were infected with the strains 4 Ma and 211 in dosages of 1000 and 10 MLD. The infecting agents were introduced subcutaneously in a total volume of 0.2 ml each day. In each group we heart-bled 3 to 5 g.-pigs obtaining 3 to 4 ml of blood for a heparin solution. /See Note7. This blood was then placed into Ehrlermayer flasks or Petocka's bottles (contents of 30 to 40 ml of the medium without blood) while a portion of the blood was inoculated in quantities of 0.2 ml on 2 to 3 Petri dishes with TKOA. (The liquid media were inoculated on solid TKOA on the 3rd and 5th days, for control purposes.) Results are shown on Table 5.

(/Note7: 1/2 pro of the commercial preparation of Heparin Spofa).

With the liquid medium we have achieved a higher percentage-number of Past tularensis isolations than on agar media.

From Tables 4 and 5 we can conclude that our modified liquid medium has good cultivating and isolating characteristics and is suitable

even for diagnostic purposes especially when larger amounts of the suspected material to be tested are used as inocculum (9).

Discussion

The difficulties which we encounter in the cultivation of Past. tularensis on a liquid medium are frequently ascribed to the great demands of the microbe and the presence of several factors necessary for growth (1, 2, 5, 12, 14, 19, 20, 21). An interesting contribution is the work of Steinhaus, et al (17) and especially of Snyder, et al (16) who succeeded in the cultivation of several strains on a simple medium containing peptone, NaCl and glucose. Our experiments fully confirmed his data. The success of the cultivation, however, frequently depends on the selection of suitable -- high quality peptone (16, 8). An important factor, therefore, will be the method of preparation of the peptone and its substitutes (1, 4, 20, 21) and possibly also the relationship of the components contained in the peptone -- growth stimulating and growth inhibiting ones -- as pointed out by Knothe, et al (8) for Sityphi.

Summary

Cultivation of Pasteurella tularensis in a Simple Liquid Medium

(1) The possibility of cultivating Pasteurella tularensis on a simple medium, containing bactopeptone (Difco or Organofarma), glucose and NaCl, was confirmed. Growth on this medium depended on the size of the inocculum and the quantity of peptone contained in the medium. Beginning with a certain concentration of both peptones tried, an inhibition of growth was observed (Organofarma-- 1%, Difco -- 2%). The Organofarma peptone had inferior growth properties than bactopeptone Difco.

(2) The cultivation conditions were favorably influenced by an addition of sodium thioglycollate (in the optimum proportion to the concentration of peptone) and particularly by an addition of 2-5% difibrinated rabbit blood. Enrichment of the medium with the above constituents intensified growth and permitted to reduce the inocculum on both peptones even to single microbes.

(3) A simple liquid medium was prepared -- TKGL (sodium thioglycollate, blood, glucose and liquid). The qualities of this medium for cultivation and isolation were tested against the solid media. The liquid medium was found very good in these experiments.

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